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—Original Article—

Dynamics of CD3$^+$ T-cell Distribution Throughout the Estrous Cycle and Gestation in the Bovine Endometrium

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Abstract. T cells are the dominant lymphocytes in the endometrium and are considered to play a crucial role in implantation and in the maintenance of gestation through cytokine production and immune regulation. The mechanisms underlying immunoregulation at the feto-maternal interface are still obscure for this complex system. Understanding the role of T cells is a key factor in understanding the endometrial immune system. In this study, the distribution of endometrial CD3$^+$ T cells in bovines was examined by immunohistochemical analysis. The estrous cycle and gestation was divided into 4 stages, and the number of CD3$^+$-positive T cells was counted in each stage. CD3$^+$ cells were found in the endometrium in significant numbers throughout the estrous cycle and were mostly located in the subepithelial area. The number of CD3$^+$ cells significantly increased in the early and mid-luteal phases but decreased after implantation with the progression of gestation. No T cells were found in the placentome or specifically in the tissues near the fetus, including the trophoblastic area. In addition, very few T cells were found in stromal regions close to the myometrium of the endometrium. These findings suggest that downregulation of bovine endometrial CD3$^+$ T-cell functions is closely related to the successful maintenance of gestation in a spatiotemporal manner.

Key words: Bovine endometrium, CD3$^+$ T cell, Estrous cycle, Gestation, Placentome

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The endometrial immune system displays specific features that are distinct from those of other typical mucosal sites because it is uniquely adapted to facilitate specialized physiological functions, including the estrous cycle, fertilization, implantation, pregnancy and parturition [1]. Successful mammalian pregnancy depends upon the tolerance of a genetically incompatible fetus by the maternal immune system.

Endometrial leukocytes consist of various T cells, natural killer (NK) cells, neutrophils, monocytes and B cells [2]. In the endometrium, T cells constitute 50–60% of all lymphocytes before pregnancy [3]. The lymphocyte composition in the human endometrium changes during the menstrual cycle [4], but there has been disagreement on the dynamics of endometrial T cells during the menstrual cycle [3]. In decidual tissue, approximately 5–20% of CD45$^+$-positive lymphocytes are T cells during early gestation, and the proportion increases to 40–80% as gestation progresses [5]. Although many studies have investigated T-cell subsets in human endometrial and/or decidual tissues, their *in vivo* functions are still unclear [6, 7].

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In ruminants, as in other species, peripheral and endometrial leukocyte numbers fluctuate with time during gestation [8]. The number of these cells significantly decreases between early and middle gestation in the endometrium of cattle [9] and sheep [10–12]. By the middle of gestation, virtually no lymphocytes or macrophages are found in the caruncle, and these cells are limited to the intercaruncular epithelium [13, 14]. The caruncles are the sites where the fetal chorion comes into close contact with the maternal endometrium to form placentomes; therefore, these findings suggest that maternal immune tolerance is spatiotemporally controlled in the area adjacent to fetal tissues. Studies have been performed on the distribution of endometrial T cells during the estrous cycle and early gestation in cattle [15, 16], but understanding of the mechanism underlying immunotolerance during gestation is still limited [8, 17]. T cells are the dominant lymphocytes in the endometrium and are considered to play a crucial role in implantation and the maintenance of gestation through the production of cytokines and immune regulation [18]. Therefore, accumulating data regarding endometrial T-cell distribution is necessary for understanding the bovine endometrial immune system.

The purpose of this study was to determine the bovine endometrial T-cell distribution throughout the reproductive cycle. We detected endometrial T cells immunohistochemically by using an anti-CD3 antibody, and the spatiotemporal T-cell distribution was analyzed quantitatively throughout the estrous cycle and gestation.

Materials and Methods

Animals and tissue collection

Endometrial and placentomal tissues were collected at various stages during the estrous cycle and gestation from Japanese black and Holstein cows in a local abattoir and the National Institute of Agrobiological Sciences (NIAS), Japan. The stage of the estrous cycle was estimated from the gross appearance of the ovaries and uterus [19], and the gestation date was estimated from the crown-rump length [20]. The stages of the cycle were designated as the early luteal (stage I; n=3), middle luteal (stage II; n=3), middle-to-late-luteal (stage III; n=3) and follicular (stage IV; n=3) phases. In pregnant cows, the stages of gestation were designated as the peri-implantation period (stage PI; n=3), early gestation (stage EG; days 50 to 90, n=3), mid-gestation (stage MG; days 100 to 180, n=3) and late gestation (stage LG; days 210 to 270, n=3). The stage PI samples were collected from a designated pregnant cow that had been subjected to artificial insemination at the NIAS. The day of insemination was designated as day 0 of gestation, and the PI samples were collected on days 19, 20 and 25 of gestation. Endometrial and placentomal tissues were collected and fixed in 10% formaldehyde (pH 7.4). The postfixed tissues were dehydrated in alcohol and xylene and embedded in paraffin wax. The embedded samples were cut into 5 μm sections with a rotary microtome and placed on slides (Matsunami, Kishiwada, Japan) overnight at 40 C. All animal care and experimental procedures were carried out in accordance with the guidelines of the Animal Care and Use Committee of the Iwate University and NIAS.

Immunohistochemistry

Anti-CD3 (MM1A, diluted 1:100; VMRD, Pullman, WA, USA) monoclonal antibody, which reacts specifically with an antigen molecule on the surface of the bovine TcR/CD3 complex, was used to detect T cells [21]. Tissue sections were permeabilized for 20 min with 0.3% hydrogen peroxide in methanol and washed three times in phosphate-buffered saline (PBS, pH 7.4). They were then incubated with PBS containing 1.5% normal donkey serum and 0.1% Triton X-100 for 30 min and incubated with the primary antibody in 0.1% Triton X-100/PBS overnight at 4 C; 0.1% Triton X-100/PBS was used for the controls. After the tissue samples were washed 6 times in PBS, they were incubated with Histofine Simple Stain MAXPO (M) (Nichirei, Tokyo, Japan) as a secondary antibody for 30 min at room temperature. Subsequently, they were washed 3 times in PBS and incubated in 3,3-diaminobenzidine tetrahydrochloride (0.2 mg/ml, pH 7.5) for 5 min at room temperature. Counterstaining was performed using hematoxylin (Merck KGaA, Darmstadt, Germany). The slides were photographed using an ECLIPSE E600 microscope with an attached Digital Sight camera (Nikon, Tokyo, Japan).

Quantification of $CD3^+$ cells

The number of cells in the digital images (200-fold magnification) was counted using the ImageJ software. The numbers of $CD3^+$ cells from the digital images were expressed per 0.1 μm^2 tissue area. The cells were counted in at least three different intercaruncular areas in each slide, and the numbers of $CD3^+$ cells per 0.1 μm^2 were averaged. All the scores are presented as mean ± standard error (SE) values.

Statistics

Data were analyzed using the JMP software (SAS Institute, Cary, NC, USA) with one-way ANOVA followed by a Tukey-Kramer multiple comparison test. P-values of <0.05 were considered significant.

Results

$CD3^+$ cells were detected as positive signals around their nuclei (Fig. 1A'), and significant numbers of these cells were observed throughout the estrous cycle in the stratum compactum, that is, the sublayer of the stratum functionalis facing the interior of the uterus (Fig. 1). During very early gestation, the endometrial distribution was similar to that seen during the estrous cycle, but the number of cells decreased with the progression of gestation. Furthermore, in the placentome, specifically in the tissues near the fetus, including the trophoblastic area, no T cells were found. In addition, a certain number of positive cells were detected in the lower part of the caruncle close to the area with the highest density of blood vessels, which was near the myometrium (Fig. 2).

The notable changes in the number of $CD3^+$ cells in the intercaruncular endometrium during the estrous cycle and gestation are shown in Fig. 3. The number of $CD3^+$ cells was higher during stages I–III of the estrous cycle than during stage IV, but the differences were not statistically significant. A similar number of cells was detected until early gestation. However, during mid to late gestation, the numbers of $CD3^+$ cells were significantly lower than those seen during stages I–III, and this reduction in the number was seen until late gestation.

Discussion

Dynamic changes in T-cell distribution were detected immunohistochemically in the bovine endometrium during the estrous cycle and gestation. We found that the number of T cells decreased during late gestation. This finding suggests a new aspect of the T cell role: a regulatory function for endometrial coordination with embryonic information, which is limited around the time of implantation in cows. In the present study, we examined the endometrial T-cell distribution throughout the reproductive cycle, namely, during the different stages of the estrous cycle and gestation; this aspect has not been studied previously. An additional refinement was the use of a T cell-specific marker antibody rather than a pan-leukocyte marker antibody or morphological features, as used in previous studies [9, 13–15].

Immunological endometrial and immune cell functions are crucial factors in the establishment of implantation and placentation, and therefore, various studies have been carried out in humans and rodents; less work has been done on T cells in cows throughout the estrous cycle and gestation. In previous studies, bovine endometrial lymphocytes were detected, although their numbers decreased from early to mid gestation, and no lymphocytes were detected in the caruncular endometrium [9, 14]. Generally, the detection of $CD3^+$ T cells in the current study was in agreement with previous reports: cell numbers decreased as gestation progressed, and no T cells were found in the caruncle. However, specifically in cows, the placentome develops in the caruncular area, and no T cells were

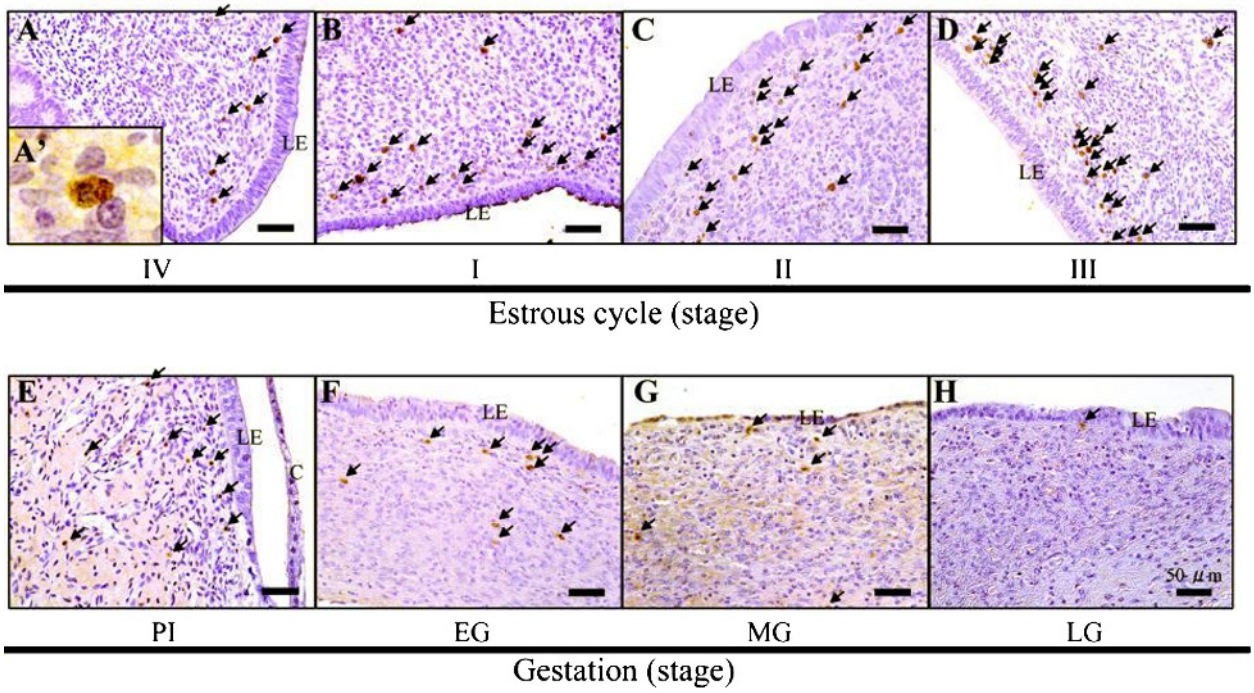

Fig. 1. Localization of CD3+ cells in the endometrium during the estrous cycle and gestation. Immunohistochemistry was used to detect CD3+ cells in the endometrium during the estrous cycle (A–D and A') and gestation (E–H). The arrows show CD3+ cells. (A) stage IV, (B) stage I, (C) stage II, (D) stage III, (A') stage IV in an enlarged portion of the frame shown in A, (E) peri-implantation period (PI), (F) early gestation (EG), (G) mid-gestation (MG), and (H) late gestation (LG). Scale bars=50 μm. LE, luminal epithelium; C, conceptus.

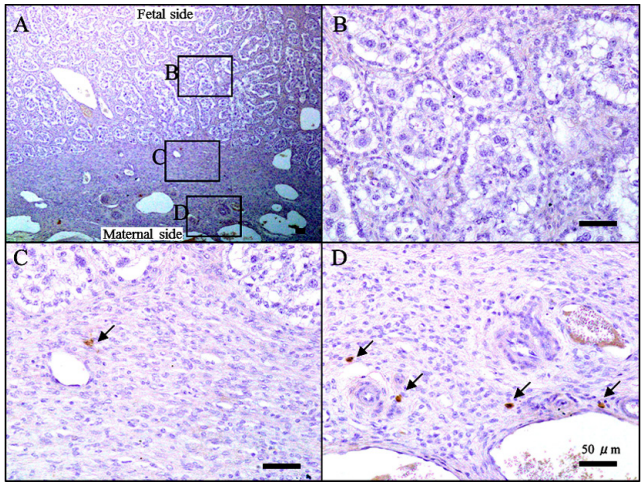

Fig. 2. Localization of CD3+ cells during mid-gestation in the placentome. Immunohistochemistry was used to detect CD3+ cells in the placentome during mid-gestation. (A) A 40-fold magnified image of the placentome. The top and bottom of (A) show the fetal side and maternal side, respectively. (B)–(D) These are 200-fold magnified images of the frames shown in (A). The arrows show CD3+ cells. Scale bars=50 μm.

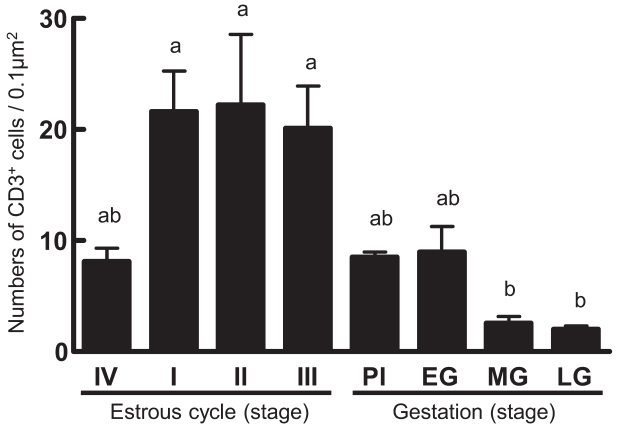

Fig. 3. Number of CD3+ cells in the intercaruncular endometrium during the estrous cycle and gestation. The number of CD3+ cells detected by immunohistochemistry was counted in endometrial sections. The values indicate the number of CD3+ cells per 0.1 μm2 area and are shown as mean ± SE values. Different letters above bars indicate significant differences at $P<0.05$. PI, peri-implantation period; EG, early gestation; MG, mid-gestation; LG, late gestation.

found there, while a small number of T cells were recognized in the intercaruncular area, and this number decreased with the progress of gestation. Considering this and the endometrial expression of T cells during the estrous cycle, the localized disappearance of T cells may be necessary for the maintenance of gestation in bovines. As shown in Fig. 1, T cells were equally numerous throughout the endometrium, with no difference between the caruncular and intercaruncular epithelium in this regard. During the estrous cycle, however, there were some discrepancies between our findings and those of previous studies. Although the current study showed that the number of T cells was higher in stages I and II than in stage IV, Cobb and Watson [15], using anti-CD5 antibody immunohistochemistry, reported that the number of T cells increased during stage IV. CD5 may be expressed in T cells, but it is also expressed by B cells and endothelial cells in the endometrium [13, 22], whereas CD3 antibody recognizes a specific T-cell marker. Therefore, this discrepancy may depend on the specificity of the antibodies used; the CD3 antibody may be more specific. CD3-positive cells may be excellent markers for investigating the immune tolerance of the endometrium. When endometrial aberration occurs, $CD3^+$ cells appear and may therefore be related to preventing placental disorder [1].

Changes in the endometrial T-cell distribution are believed to be regulated by adhesion molecules and humoral factors. In the ovine endometrium during early gestation, vascular cell adhesion molecule 1 (VCAM-1) is expressed, and differences are seen between the VCAM-1 expression in the caruncular and intercaruncular endometrium [22]. Furthermore, it is considered that humoral factors, including hormones, cytokines and chemokines are involved in the infiltration and migration of lymphocytes [23]. There is a close association between endometrial leukocytes and decidualization [24], and our results indicate that the T-cell distribution in the bovine endometrium differs from that in the human endometrium [5]. These differences are possibly related to the structural variations in the placenta, specifically the epitheliochorial and hemochorial placenta. In the former, the placental barrier includes an intact uterine epithelium, but the latter is the most invasive form of placentation, as there is loss of all the maternal tissue including the endothelium of the maternal blood vessels at the site of placentation, and maternal blood irrigates the fetal membrane directly. Therefore, although endometrial T cells play a coordinating role at the feto-maternal interface, different functions may be involved in cattle and humans through changes in T-cell distribution and humoral regulation.

Although little is known about the *in vivo* functions of endometrial T cells, the appearance of T cells may be related to feto-maternal interactions. In somatic cloned cows, T-cell expression seems to indicate placental function abnormalities involving major histocompatibility antigen complex (MHC) class I expression, and the number of T cells has been reported to be significantly higher than that in control [25]. T cells contribute to the placental functions in many species because the emergence of T cells is closely related to abortion [26] and placental retention [27]. Furthermore, maternal lymphocytes promote tissue remodeling in stimulating endometrial angiogenesis [28]. Immunological regulation during gestation is still a disputed issue, but T cells play a crucial role in coordination between the mother and fetus, especially during the implantation period when endometrial tissue remodeling through lymphocyte functions is necessary for fecundity. Further research is required to investigate endometrial T-cell subsets and to determine the role of each subset in the bovine endometrium.

In conclusion, the dynamics of the bovine endometrial T-cell distribution were determined throughout the estrous cycle and gestation. The number of T cells was higher in the early-mid luteal phase, but after implantation, T cell numbers decreased as gestation progressed. No T cells were found in the placentome. The T-cell distribution might contribute to implantation and endometrial tissue remodeling. Thus, bovine endometrial T cells might be closely related to the success and maintenance of bovine gestation in a spatiotemporal manner.

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